Use of immunocytochemical staining to identify cells in peritoneal fluid and washings at laparoscopy and laparotomy

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SUMMARY Specimens of peritoneal fluid or peritoneal washings from a series of 106 patients who had had laparotomy or laparoscopy for gynaecological complaints were studied “blind” by conventional cytology and immunocytochemical staining. The antibodies used were Ca 1 or Ca 2, anti-CEA, and HMFG-2 or E29. All these are directed against epithelial antigens and are expressed on most malignant epithelial cells and weakly or not at all on mesothelial cells. It was hoped that these reactions would confirm diagnoses made by conventional cytology and possibly show malignant cells which had not already been identified.

Of 28 patients with malignant disease (chosen to exclude any with frank ascites), eight gave positive immunochemical reactions, only four having been reported positive from conventional examination. Of 77 patients without malignant disease, HMFG-2 or E29 gave positive reactions in seven, Ca 1 or Ca 2 in two, and anti-CEA in two (reactions with plasma cells being disregarded). Some misleading reactions were probably due to endometrial cells.

It was concluded that the antibodies used in this study are not sufficiently specific or sensitive to allow immunocytochemical staining to replace conventional cytological diagnosis but are a useful supplementary aid.

In recent years it has become a widely recommended practice to obtain peritoneal washings or aspirated pelvic fluid for cytology when laparotomy is performed in cases of gynaecological cancer. If malignant cells are reported this is an indication that more aggressive treatment should be carried out. Unfortunately, identifying small numbers of malignant cells in this type of material is extraordinarily difficult, a fact that is not properly reflected in published reports. We investigated the possibility of applying immunocytochemical staining, using monoclonal antibodies, in the hope that the reactions would show small numbers of tumour cells that might otherwise have been overlooked and help distinguish benign from malignant cells when the morphology was equivocal.

Material and methods

A series of 106 patients with a wide range of both

benign and malignant gynaecological conditions were selected for study. In all the patients the diagnosis was unknown to the cytologist. The results, however, were given to the clinician if malignant cells were suspected or diagnosed. Patients undergoing primary surgery for ovarian cancer were not studied. In those patients with ovarian cancer who were undergoing a second look procedure none was selected for study who already had macroscopic disease present. In one patient histologically confirmed retroperitoneal disease only was present. Of the patients with cervical cancer, only one might have had an intraperitoneal tumour. Of those with endometrial cancer, two had metastatic disease in the ovaries, while in another who had had breast cancer treated in the recent past malignant deposits were found on the bowel and in the liver. Patients with endometriosis were particularly sought out as, theoretically, endometrial cells could be shed from these lesions into the peritoneal fluid, and these might be difficult to distinguish cytologically from adenocarcinoma cells.

An initial attempt was made to aspirate free peritoneal fluid in all patients. Only when this was unsuccessful was washing of the pelvis undertaken

with isotonic saline. In those patients submitted to laparotomy particular attention was paid to haemostasis during incision of the abdominal wall. Sufficient peritoneum was then opened to permit insertion of a Morris retractor to display the pelvic cavity and the lower paracolic gutters. Any fluid present was aspirated with a 10 ml syringe and quill and immediately transferred to a 30 ml container with 20 mg edetic acid as anticoagulant. In those patients undergoing laparoscopy fluid was aspirated from the pouch of Douglas using a Verre's needle and similarly transported to the laboratory.

In 11 patients undergoing laparotomy insufficient free fluid was obtained. In these women 100–200 ml isotonic saline was introduced into the pelvic cavity. After two to three minutes as much fluid as possible was recovered using a syringe and quill and transported to the laboratory in containers with anticoagulant, as described above. Smears were made from the centrifuged deposit. At least one was wet fixed and stained by the Papanicolaou routine. The rest were air dried, and of these, at least two were stained with May-Grünwald and Giemsa, the remainder being used for immunocytochemical staining.

MONOCLONAL ANTIBODIES

Antibody Ca 1 This was kindly provided by Wellcome Diagnostics. This antibody is directed against a cancer associated antigen that has been detected on a range of different neoplasms.¹ Its use in the detection of malignant cells in serous fluids has been described previously.^{2–4} Reactions are rarely seen with mesothelial cells, and even more rarely with plasma cells.

Antibody Ca 2 This was kindly provided by Professor Henry Harris. It is directed against a similar determinant to Ca 1. Its reactions on serous fluid cells have recently been described.⁵

Anti-CEA antibody This was kindly provided by Dr JRF Corvalan and Dr C Ford. The reactions of this monoclonal antibody with normal and malignant gastrointestinal epithelium and with breast carcinomas in cryostat sections have been described,⁶ as well as its reactions with malignant cells in effusions.⁴ Reactions with mesothelial cells are rarely seen, if ever, but plasma cells occasionally stain strongly.

Antibody HMFG-2 This was kindly provided by Dr J Taylor-Papadimitriou. It is directed against a determinant in the membrane of human milk fat globules and reacts with a variety of carcinomas, as well as giving weaker reactions with certain other epithelial cells and occasional mesothelial cells.⁷

Antibody E29 This was made in the author's (AG) laboratory.⁸ This antibody is directed against a human milk fat globule membrane antigen and is found on a variety of carcinomas, including those of ovary and endometrium. The antigen is also weakly

expressed on normal epithelial cells and occasionally on mesothelial cells. The reactions of this antibody are similar to those of HMFG-2.^{6,8,9}

IMMUNOCYTOCHEMICAL STAINING TECHNIQUE

Air dried smears were fixed with acetone and methanol (50/50 v/v) for five minutes and then stained by an immunoalkaline phosphatase staining procedure.^{4,10} Briefly, this procedure entailed successive incubation with primary mouse monoclonal antibody (suitably diluted), rabbit antimouse immunoglobulin, and alkaline phosphatase-mouse monoclonal anti-alkaline phosphatase (APAAP) immune complexes. Slides were washed in Tris buffered saline (pH 7.6) between each incubation step. The alkaline phosphatase reaction product was visualised using naphthol As Mx phosphate and fast red as substrates. Endogenous alkaline phosphatase was inhibited by adding 1 mM levamisole to the substrate solution. The optimum dilution for each primary monoclonal antibody was established by titration on cell smears containing morphologically recognisable malignant cells from patients with breast or ovarian carcinoma, or both.

CLASSIFICATION

In all cases conventionally stained smears were examined along with the treated slides. This comparison together with the morphology of stained and unstained cells in the antibody treated smears allowed an opinion to be formed of the identity of the reacting cells. Where serious doubt existed the cells were classified as "unidentified."

Results

Tables 1 and 2 show the reactions observed.

BENIGN CONDITIONS

Of the 41 cases with normal pelvic findings, pelvic adhesions, or uterine fibroids only, all but four cases showed completely negative reactions. In two cases small numbers of plasma cells were stained with anti-CEA. In two other cases single clusters of unidentified cells were seen to react with E29, and in one of these cases two cells (possibly mesothelial) reacted with anti-CEA.

Of 24 cases with endometriosis or adenomyosis, 19 were entirely negative. In three cases a few cells resembling mesothelial cells reacted with HMFG-2 or E29, and in one of these a single mesothelial cell reacted weakly with Ca 2. In two other cases small numbers of unidentified cells reacted with E29 and Ca 2; these could have been of endometrial origin.

Of the 12 cases with benign ovarian conditions, one showed weak staining of mesothelial cells with E29 and HMFG-2. Another, with a mucinous cyst-

Table 1 Benign conditions in patients studied

Diagnosis clinical and histological	No of cases	Cytology of Malignant cells	HMFG2/E29	Monoclonal antibodies	
				Ca1 or Ca2	Anti-CEA
Normal pelvis (n = 18)	15				p±
	1		?+		?±
	1		?+		
Pelvic adhesions (n = 12)	11				p±
	1				
Uterine fibroids only (n = 11)	11				
Endometriosis (n = 21)	16				
	2		m±		
	1		m±	m±	
	2		?±	?±	
Adenomyosis (n = 3)	3				
Benign ovarian conditions (n = 12)	10				
	1		m±		
	1 (case 2)	+	t+		t+
Granulosa cell tumour (n = 1)	1 (case 1)	?+*	?+		?+ +

*On review.

p = cells resembling plasma cells; m = cells resembling mesothelial cells; t = cells resembling tumour cells; ? = unidentified cells; ++ = more than 20 per slide; + = 5–20 per slide; ± = less than 5 per slide.

tadenoma, gave misleading positive results. There was also one case of granulosa cell tumour giving positive reactions, which were probably not due to tumour cells. It is noteworthy that both of these cases contained areas of endometriosis in the pelvis.

MALIGNANT CONDITIONS

Of 28 cases with malignant disease, treated or untreated, eight showed many cells reacting with some or all of the antibodies. In seven of these the cells concerned resembled tumour cells. Four of them contained cells diagnostic of or suggesting carcinoma in the routinely stained smears, so that the immunocytochemistry only offered confirmation. In two malignant cells were found on review of the routine smears, and in one the identification depended on the

immunocytochemically stained smears alone. In one other case (carcinoma of the cervix) a single possibly malignant cell was found with anti-CEA antibody.

MISLEADING OR UNEXPECTED RESULTS

Case 1 This 51 year old woman presented with post-menopausal bleeding and was found to have a pelvic mass. A total abdominal hysterectomy and bilateral salpingo-oophorectomy was performed for a 450 g left ovarian tumour. There was a small volume of free fluid which was submitted for study. The major part of the lesion comprised a granulosa cell tumour. The small cells with hyperchromatic nuclei were arranged in solid and trabecular areas with loosely arranged pale fibrous stroma intervening. There was also an area of endometriosis with associated haemorrhagic

Table 2 Malignant conditions in patients studied

Diagnosis clinical and histological	No of cases	Cytology of Malignant cells	HMFG2/E29	Monoclonal antibodies	
				Ca1 or Ca2	Anti-CEA
Carcinoma endometrium (n = 8)	5				
	1	±*	t+		t+
	1	+	t+	t+	t+
	1	+	t+	t+	
Carcinoma ovary (n = 10)					
Active	2				
Borderline	1	+	t+	t+	t+
Second look	6				
	1	suspected	t+	t+	
Carcinoma cervix (n = 8)	5				—
	1				?±
	1 (case 3)		t+		t+
	1 (case 4)	* suspected	t+		
Sarcoma of uterus (n = 2)	2				

*On review.

p = cells resembling plasma cells; m = cells resembling mesothelial cells; t = cells resembling tumour cells; ? = unidentified cells; ++ = more than 20 per slide; + = 5–20 per slide; ± = less than 5 per slide.

cysts. The right ovary also contained an area of endometriosis. The endometrium contained a polyp among focal cystic hyperplasia.

Conventionally stained smears were not considered to have shown evidence of carcinoma. With immunocytochemical staining, some unidentified cells reacted with HMFG-2, E29, and anti-CEA. They occurred in clusters and, on review, could be seen in the conventional smear. They differed from mesothelial cells but did not suggest granulosa cell tumour. These may well have been endometrial cells.

Case 2 This 31 year old patient presented with acute abdominal pain. At laparotomy extensive adhesions were present, in addition to nodules of endometriosis. Bilateral ovarian cysts necessitated a left salpingo-oophorectomy and right ovarian cystectomy. Histology confirmed bilateral endometriotic cysts with a single mucinous cystadenoma in the right ovary.

Free fluid was examined by cytology. With conventional staining, clusters of cells were found that were interpreted as having come from an adenocarcinoma. They reacted with anti-CEA and E29 but not with Ca 2. It is possible that these cells were, in fact, endometrial.

Case 3 This 45 year old patient was treated by caesium applications and radical hysterectomy with bilateral pelvic lymphadenectomy for a clinical stage 1B moderately differentiated adenocarcinoma of the cervix. At laparotomy a considerable degree of fibrosis was found between the cervix and rectum from which a biopsy specimen was taken and submitted for frozen section. There was no other evidence of palpable metastatic disease. The frozen section examination was reported to show only fibrosis, but subsequent paraffin section suggested that a metastatic deposit was present, although a certain diagnosis was not possible. Bilateral pelvic lymph node metastases were present. Pelvic irradiation was given. Twenty three months after treatment had started a pelvic recurrence with a right hydronephrosis was confirmed, and external irradiation was given. Conventionally stained smears of peritoneal fluid deposit showed no cells suspicious of carcinoma. In the immunocytochemically stained smears, however, a number of cells of malignant appearance gave strong positive reactions with anti-CEA and with HMFG-2. Reactions with Ca 1 were negative.

Case 4 This 33 year old woman presented with a moderately to poorly differentiated squamous cervical carcinoma. Although the cervical lesion was very small indeed (0.5×1 cm), the lymph nodes of the left inguinal, bilateral external, and common iliac and para-aortic groups were extensively affected. There were no obvious peritoneal seedlings. Despite total abdominal hysterectomy, pelvic irradiation, and systemic chemotherapy she died within six months.

Immunocytochemical reactions of the peritoneal cells were negative with Ca 1 and anti-CEA, but E29 showed a group of five positive cells that were morphologically consistent with carcinoma. Conventional examination was initially negative for malignant cells. On review one dubious cluster of cells was found, not amounting to good evidence of carcinoma.

Discussion

CONVENTIONAL CYTOLOGICAL DIAGNOSIS

Keettell and Elkins in 1956¹¹ first advocated the cytological examination of peritoneal washings for the assessment of tumour cell dissemination at operation for carcinoma of the ovary. Since then the same approach, as well as the examination of free fluid from the pouch of Douglas, has become part of the staging protocol for ovarian cancer recommended both by the International Federation of Gynaecology and Obstetrics (FIGO) and the International Union Against Cancer.¹² It is also recommended during laparotomy for endometrial and cervical carcinoma.

In our experience this material is particularly difficult to interpret. Peritoneal washings are apt to be contaminated with blood and shreds of detached mesothelium, and it is difficult to avoid both positive and negative mistakes. With few exceptions,¹³ workers in this area have either done without negative controls, or else reported unacceptably high false positive rates in patients without cancer.

USE OF IMMUNOCYTOCHEMISTRY

Because of these uncertainties, it is highly desirable to have other more objective tests for small numbers of free malignant cells in the peritoneum. At this time the most hopeful advance is the use of immunocytochemical staining. Coleman and Ormerod¹⁴ briefly reported on a small series of peritoneal washings from patients with ovarian cancer who had cytotoxic treatment, using a polyclonal antiserum against a marker CX-1, and they illustrate a group of labelled cells that seem to be typical of adenocarcinoma.

The present study was intended to assess the value of this approach using some monoclonal antibodies already shown to be of value in the diagnosis of cancer cells in pleural and peritoneal effusions.^{4,5} The results confirm that in cases with otherwise identifiable malignant cells in fluid aspirated from the pelvis the antibodies react as expected.

Only a very small proportion of the cases in this series could have been expected to contain malignant cells. In two of the six patients in whom it was finally agreed that tumour cells were present the relevant cells would have been missed if immunochemistry had not been used. Our findings were disappointing, how-

ever, in that some positive reactions were observed in cells that were not thought to be malignant, and it is clear that the immunocytochemical reactions cannot be used as substitute for ordinary cell recognition. Their value is to provide additional information to an observer with experience of the cytology of peritoneal fluid.

When cells are found that could be malignant but which cannot be confidently identified, the immunocytochemical reactions still do not furnish proof, because of insufficient sensitivity and specificity. The most troublesome problem is that of clusters of tightly packed cells. Even if their arrangement is unlike that of mesothelial cells, the possibility has to be considered that they are benign cells from endometrium. The finding of recognisable endometrial cells is rare in ascitic fluid obtained by paracentesis, but less so in fluid obtained from the pouch of Douglas by culdocentesis or at laparotomy.^{15 16} This could be an important source of "false positive" error, though only recently emphasised in the published findings on this subject.^{17 18}

To find out the reactions of benign endometrial cells with the antibodies used in this study the same panel was applied to smears made from nine samples of endometrium obtained by curettage. Although most of the endometrial cells were unstained in all samples, there were five that contained cells reacting with E29 and five that reacted with anti-CEA (two, if cells of apparently squamous origin are excluded). This is of particular interest in relation to the two misleading cases for which positive results were found in patients with benign lesions. Although each had an ovarian tumour, both also had histologically confirmed endometriosis.

In conclusion, we find that the antibodies used have insufficient sensitivity or specificity to replace the conventional cytological examination of fluid aspirated from the pelvis, but they provide more information than Giemsa and Papanicolaou stains alone. When tumour cells are scarce, a low power scan of immunocytochemically stained slides can alert the observer to cells which would otherwise be missed, and for this reason it is useful to examine them before spending time on the "conventional" slides. Carefully aspirated free fluid is much easier to interpret than saline washings.¹⁹ The possibility of false positive results when endometriosis is present must be seriously considered in future studies in this field.

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